Iranian Journal of Veterinary Science and Technology
Vol. 5, No. 1, 2013, 26-34

IJVST

Effect of follicle stimulating hormone and testosterone on viability rate of cryopreserved spermatogonial stem cell after thawing

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Received: April 21, 2012 Accepted: February 06, 2013

Abstract

Stem cells are generally defined as clonogenic cells capable of both self-renewal and differentiation. Probably the best method for long-term preservation of spermatogonial stem cells is cryopreservation. In this study, effects of Follicle Stimulating Hormone and Testosterone on viability rate of cryopreserved spermatogonial stem cell after Thawing were investigated. Sertoli and spermatogonial cells were isolated from 3-5 months old calves. Co-cultured sertoli and spermatogonial cells were treated with Follicle Stimulating Hormone and Testosterone in treatment groups before cryopreservation. Results indicated that Follicle Stimulating Hormone increased viability rate of cryopreserved spermatogonial cells in comparison with Testosterone and control group. In conclusion, using Follicle Stimulating Hormone provided proper bovine spermatogonial stem cell culture medium for *in vitro* culture and cryopreservation of these cells.

Keywords: Cryopreservation, Bovine, FSH, Testosterone

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Introduction

Spermatogenesis is a complex developmental process that originates from Spermatogonial Stem Cell (SSC). This process consists of sequential, highly organized steps of cell proliferation and differentiation resulting in generation of functional spermatozoa (McLean *et al*., 2002).

In rodents, the A single (As) spermatogonia are considered the stem cells of spermatogenesis (Huckins, 1971; Oakberg, 1971 and de Rooij., 1973). Upon division of the As spermatogonia, the daughter cells either separate each other and become two new stem cells, or stay together through an intercellular bridge and become A-paired (Apr) spermatogonia. The Apr spermatogonia develop into chains of four, eight or 16 A-aligned (Aal) spermatogonia. The Aal spermatogonia differentiate into A1 spermatogonia and after six mitotic divisions differentiate to A2, A3, A4 and B spermatogonia, which give rise to spermatocytes at the last mitotic division. Regarded to species-specific differences, in bovine testis, a comparable classification with another terminology has been reported (Wrobel*et al*., 1995). In this species, spermatogonial precursor cells have been divided into basal stem cells aggregated spermatogonial precursor cells and committed spermatogonial precursor cells. According to this classification, As–Apr spermatogonia, Aal spermatogonia and A1–A4 differentiating spermatogonia are the result of spermatogonia stem cell differentiation, respectively. Hence, in bulls, Apr spermatogonia are also thought to have stem cell properties (Wrobel *et al*., 1995). Adult mammalian testis, has multiple generations of germinal cells, therefore purification of spermatogonia is more difficult than before puberty. Bellve *et al*. (1977) obtained 90% pure fraction of type A spermatogonia from immature mice. Izadyar *et al*. (2002b) concluded that when testis from 5-month-old calves were used, approximately 1 $\times 10^6$ type A spermatogonia per gram of testis with purity about 75% could be obtained routinely.

Of all the hormones implicated in spermatogenesis, follicle-stimulating hormone (FSH) has been suggested to play a determinant role in the survival of germ cells in addition to increase spermatogonia proliferation (Baarends and Grootegoed, 1999). FSH causes an increase in SCCs colonization *in vitro* co-culture (Narenji Sani *et al*., 2012a). Testosterone, directly produced by Leydig cells in response to luteinising hormone (LH), indirectly produced in response to FSH or supplied exogenously, has been shown to be a necessary prerequisite for the maintenance of an established spermatogenesis in the adult mammalian testis (Huang and Nieschlag, 1986 and Lejeune *et al*., 1996). Testosterone and FSH have been reported to act synergistically in the adult rat by either stimulating germ-cell development and/or inhibiting germ-cell degeneration (Huang and Nieschlag, 1986). Addition of testosterone to the SCCs Co-culture has not increased SCCs colonization (Narenji Sani *et al*., 2012b).

In studies on spermatogonial isolation and purification, the availability of markers that can conclusively establish the identity of the spermatogonia is essential. In bovine, Oct-4 can be used as a specific marker for spermatogonia in the testis (unpublished data).

These SCCs should preserve for using in treatment of infertility and research study. In principle, these spermatogonia can be preserved in 2 ways, long-term culture or cryopreservation. Probably the best method for long-term preservation of spermatogonial stem cells is cryopreservation. In study of Izadyar and coworker an optimal cryopreservation protocol for bovine type A spermatogonia was developed (Izadyar *et al*., 2002a).

Consequently, the aim of the present study was to determine the effect of Follicle-Stimulating Hormone and Testosterone on viability rate of cryopreserved spermatogonial stem cell after Thawing.

Materials and methods

Testicular biopsies were obtained from 3 to 5 months old calves undergoing the TESE procedure. Obtained testis pieces were mechanically minced and floated in DMEM containing 1 mg/ml collagenase, 1 mg/ml Trypsin, 1 mg/ml hyaluronidase type II and 5 µg/ml DNase I and then incubated at 37°C for 60 min (van Pelt *et al*., 1996). After three times of washing in DMEM and excluding the interstitial cells, for secondary digestion step, seminiferous tubules were incubated in DMEM containing collagenase, hyaluronidase and DNase for 45 min. Finally, obtained cellular suspension was centrifuged at 30 g for 2 min to achieve favorite cell population. Then, spermatogonial cells were co-cultured with sertoli cells for 13 days. For sertoli cell collection, 5 µg/ml Datura stramonium agglutinin lectin (Sigma) in TBS was poured into the sterile flasks. Cells obtained from secondary enzymatic digestion were added to DSA-lectin coated flasks and incubated at 37 °C for 1 hr. Following the confluency of sertoli cells, spermatogonial cells co-cultured in seven groups, for 13 days. For co – culture of these cells, DMEM with 10% FBS, 100 mg/ml GDNF, 100 u/ml penicillin and 100 mg/ml streptomycin were used. Our experimental groups were: Control, group 1 (10 IU ml-1 FSH), group 2 (20 IU ml-1 FSH), group 3 (40 IU ml-1 FSH), group 4 (0.2 µmol L-1 Testosterone), group 5 (0.4 µmol L-1 Testosterone) and group 6 (0.8 µmol L-1 Testosterone). Culture medium plus mentioned doses of FSH and Testosterone were refreshed every 3 days.

Cryopreservation

For cryopreservation, at first the co-cultured cells were detached using EDTA–trypsin treatment (0.02% EDTA–0.1% trypsin in Ca2+- and Mg-free PBS) for 5 min at 37◦C. Then SCCs viability was assessed (see below) and the cells were transferred to cryovial and

Freezing medium was added dropwise to the cryovial containing the cell suspension during a period of 10–15 minutes, and gently mixing by inverting the vial. The freezing medium was contained: 50 % FCS, 40 % DMEM and 10 % dimethyl sulfoxide (DMSO). Slow freezing protocol was used; cryovials were placed in an insulated container at -80ºC for at least 1 day and then plunged into liquid nitrogen (-196ºC). The cells were thawed by swirling in 38ºC water bath for 30 seconds (straws) or 2 minutes (vials). The contents of the straw or vial was transferred to a tube and diluted slowly by adding two volumes, dropwise, of DMEM supplemented with 10% FCS. Then, the cells were pooled and centrifuged at 2000×gfor 5 minutes, the supernatant was removed, and the pellet was resuspended in DMEM/BSA. A sample was taken for viability assessment.

Cell viability

Cell viability was evaluated by means of the dye exclusion test (0.04% trypan blue solution).

Cells identification

For sertoli cells identification, we used Vimentin immunocytochemical staining which was described by (Anway *et al*., 2003 and Tajik *et al*., 2010). Also, Colonies of SSCs were immunocytochemically stained with anti Oct-4 (conjugated with FITC). Oct-4 has been described as a marker for undifferentiated cells (Kubota *et al*., 2004).

Colony assay

Number and diameters of spermatogonial cell derived colonies were measured on days 4, 7, 10 and 13. For the measurements we used inverted microscope (Olympus, Tokyo, Japan) equipped with ocular grid.

Statistical analysis

Results are expressed as mean±s.d. The statistical significance between mean values was determined by One Way Analysis of Variance (ANOVA) and Duncan post hoc test; p <0.05 was considered significant.

Results

Isolation and identification of spermatogonial and sertoli cells

The cell population obtained from seminiferous tubules of 3-5th month old calves testis contained mostly two cell types with different immunocytochemical features. The first cell type were proliferated and created a monolayer of cells (Figure 1), whereas the other cell type was created a colony after proliferation (Figure 2). Oct-4 is a molecular marker for SCCs identification (Figure 3). Moreover, vimentin, which is a molecular marker for sertoli cells, was detected in the feeder monolayer cells (Figure 4).

FSH and Testosterone effects on viability rate

Viability rate before freezing was > 90% for all groups and there was no significant difference between groups. But after thawing, viability rate in FSH groups was more than Testosterone and control groups ($p < 0.05$)(Table 1).

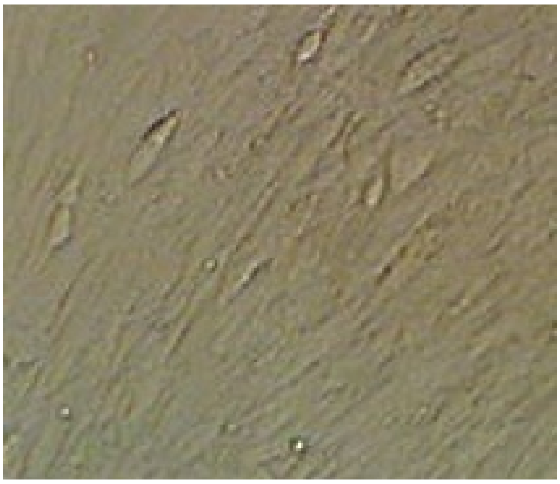


Figure 1. Sertoli cells that created a monolayer of cells.



Figure 2. The morphology of a spermatogonial derived colony that formed from co-cultured spermatogonial cells on a monolayer of sertoli cells.

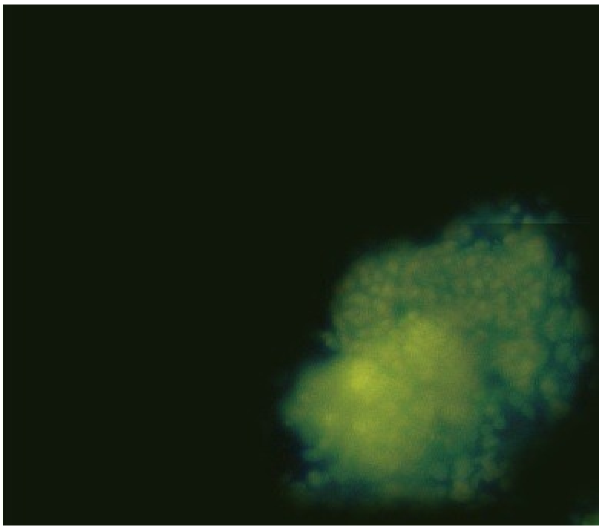


Figure 3. Oct-4 immunocytochemical staining of bovine spermatogonial stem cells.

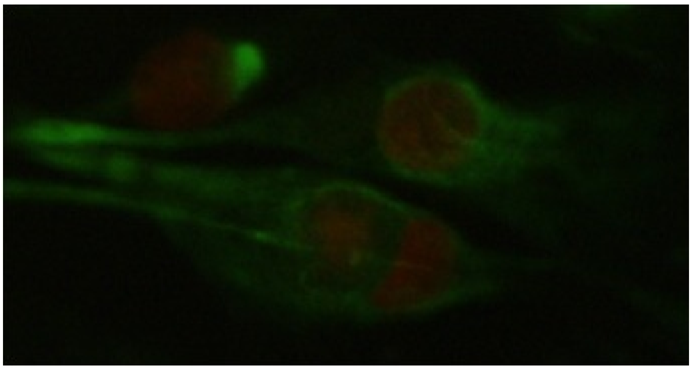


Figure 4. Vimentin immunocytochemical staining of bovine sertoli cell.

Table 1. Comparison of viability rate between control and experimental groups.

	Before Freezing	**After Thawing**
Control	91/4 %	62/7 %[b]
FSH (IU L^{-1})		
10	92/6 %	75/4 %[a]
20	93/1 %	72/3 %[a]
30	92/7 %	75/6[a]
Testosterone (µ mol L^{-1})		
0/2	92/4 %	64/7 %[b]
0/4	90/7 %	66/1 %[b]
0/8	92/7 %	64/2 %[b]

[ab]The value with different letter significantly differ within column (p<0.05).

Discussion

Suitable populations of sertoli cells and SSCs could be obtained from 3-5 month old calves, because the seminiferous epithelium of calves testis contains two distinct cell types: type-A SSCs and sertoli cells. It appears that, 3-5 month, is most appropriate age of calves for type A SSCs isolation. Most of tubule cross-sections contained type A SSCs, therefore this testis was proved to be the best source for isolation of this type of SSCs. Highly enriched populations of type A SSCs with final purity of up to 75% could be isolated routinely. Cell recovery was about 1×10^6 type A SSCs per gram of testis and the viability of isolated SSCs was always > 80% (Izadyar *et al*., 2002b). Our viability rate results are comparable to those reported for the isolation of type A SSCs from prepubertal mice (Bellve *et al*., 1977), rats (Morena *et al*., 1996) and pigs (Dirami *et al*., 1999) and bovine (Izadyar *et al*., 2002b).

Therefore, the optimum time for the recovery of sertoli cells and primitive type-A SSCs is the 5th month after birth (Izadyar *et al*., 2002b). So we used 3-5th month old calves as animal sample in our study.

In this research, isolated cells from the seminiferous tubules of 3- 5-month-old calves had two types of cells with distinct immunocytochemical feature, similar to sertoli cells and type-A SSCs. These findings are in agreement with those were reported by Koruji *et al*. (2007) who demonstrated the immunocytochemical features of sertoli cells and SSCs in mouse.

Specific marker detection of sertoli cells was vimentin immunocytochemistry staining (Anway *et al*., 2003). For confirmation of the presence of SSCs, Oct-4 was traced in the colony cells. Undifferentiated type A SSCs express Oct-4 (Kubota *et al*., 2004). So we assume that the colonies may have been largely derived from the SSCs.

Our approach was *in vitro* co-culture of sertoli cells and SSCs and determined the effects of FSH and testosterone on viability rate of cryopreserved SCCs. Proper factor was chosen for viability rate increscent of co-cultured SSCs. In FSH-treated groups, viability rate, dose regardless, was higher than that of the control and Testosterone groups.

Probably, FSH can increase the viability rate of SCCs after thawing through anti apoptosis pathways. Because in embryo, activation of this pathway through Phosphatidylinositol 3-kinase can increase viability rate. (PI3-K) (Jousan *et al*., 2008) In sertoli cells also, PI3-K was activated with FSH (Walker and Cheng, 2005). FSH can increase transferrin production via sertoli cells (Michael D, 1998) that transferrin can increase sertoli cells proliferation. Insulin like growth factor 1(IGF1) can increase cryopreserved SSCs viability rate after thawing (unpublished data). Hence FSH can increase IGF1 production from sertoli cells (Skinner, 2005), therefore this hormone can increase viability rate of SSCs.

In conclusion we postulate that FSH was more useful than testosterone in bovine for increasing of viability rate of cryopreserved SCCs.

Acknowledgments

This project was supported, in part, by a research grant from Animal Stem Cell Scientific Center of University of Tehran. Authors would like to thank Dr. P. Rahimi and Aboreihan Dairy Farm of University of Tehran.

References

Anway, M.D., Folmer, J., Wright, W.W. andZirkin, B.R.(2003) Isolation of sertoli Cells from Adult Rat Testes: An Approach to Ex Vivo Studies of Sertoli Cell Function. *Biology of Reproduction* **68**, 996-1002.

Baarends, W.M., Grootegoed, J.A. (1999) Molecular biology of male gametogenesis. Parthenon, NewYork. pp 271-295.

Bellve, A.R., Cavicchia, J.C., Millette, C.F.,

O'Brien, D.A., Bhatnagar, Y.M. andDym, M.(1977)Spermatogenic cells of the prepuberal mouse: isolation and morphological characterization. *The Journal of Cell Biology* **74**, 68-85.

deRooij, D.G.(1973)Spermatogonial Stem Cell Renewalinthe Mouse: I. Normal Situation. *Cell Proliferation* **6**, 281-287.

Dirami, G., Ravindranath, N., Pursel, V. andDym, M.(1999) Effects of Stem Cell Factor and Granulocyte Macrophage-Colony Stimulating Factor on Survival of Porcine Type A Spermatogonia Cultured in KSOM. *Biology of Reproduction* **61**, 225-230.

Huang, H.F.S. andNieschlag, E.(1986) Suppression of the Intratesticular Testosterone Is Associated with Quantitative Changes in Spermatogonial Populations in Intact Adult Rats. *Endocrinology* **118**, 619-627.

Huckins, C.(1971) The Spermatogonial Stem CellPopulationin Ault Rats. *Cell Proliferation* **4**, 313-334.

Izadyar, F., Matthijs-Rijsenbilt, J.J., Ouden, K.D., Creemers, L.B., Woelders, H. and de Rooij, D.G.(2002a) Development of a Cryopreservation Protocol for Type A Spermatogonia. *Journal of Andrology* **23**, 537-545.

Izadyar, F., Spierenberg, G.T., Creemers, L.B., den Ouden, K. and de Rooij, D.G.(2002b) Isolation and purification of type A spermatogonia from the bovine testis. *Reproduction* **124**, 85-94.

Jousan, F.D., Oliveira, L.J., Hansen and P.J.(2008) Short-Term culture of *in vitro* produced bovine preimplantation embryos with insulin-like growth factor-i prevents heat shock-induced apoptosis through activation of the Phosphatidylinositol 3-Kinase/Akt pathway. *Molecular Reproduction and Development* **75**, 681-688.

Koruji, S.M., Movahedin, M., Mowla, S.J. and Gourabi, H. (2007) Colony formation ability of frozen thawed spermatogonial stem cell from adult mouse. *Iranian Journal of Research Medicine* **5**, 109-115.

Kubota, H., Avarbock, M.R. andBrinster, R.L.(2004) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 16489-16494.

Lejeune, H., Chuzel, F., Thomas, T., Avallet, O., Habert, R., Durand, P. and Saez, J. (1996) Paracrine regulation of Leydig cells. *Endocrinology* **57**, 55-63.

McLean, D.J., Russell, L.D. and Griswold, M.D.(2002) Biological Activity and Enrichment of Spermatogonial Stem Cells in Vitamin A-Deficient and Hyperthermia-Exposed Testes from Mice Based on Colonization Following Germ Cell Transplantation. *Biology of Reproduction* **66**, 1374-1379.

Michael D, G.(1998) The central role of Sertoli cells in spermatogenesis. Seminars in Cell & *Developmental Biology* **9**, 411-416.

Morena, A.R., Boitani, C., Pesce, M., De Felici, M. andStefanini, M.(1996) Isolation of highly purified type A spermatogonia from prepubertal rat testis. *Journal of Andrology* **17**, 708-717.

NarenjiSani, R., Tajik, P., yousefi, M.H., Movahedin, M., Qasemi-Panahi, B. and Shafiei, Sh. (2012a) Follicle-Stimulating Hormone increase spermatogonial stem cell colonization during *In vitro* co-culture**.** Accepted in Veterinary Research Forum.

NarenjiSani, R., Tajik, P., yousefi, M.H. and Movahedin, M. (2012b) Effect of testosterone on spermatogonial cell colony formation during *In vitro* co-culture**.** Accepted in *Iranian Journal of Veterinary Medicine.*

Oakberg, E.F.(1971) Spermatogonial stem-cell renewal in the mouse.*The Anatomical*

Record **169**, 515-531.

Skinner, M.K. (2005) Sertoli cell secreted regulatory factors. In Sertoli Cell Biology. Elsevier Science, San Diego. pp 107-120.

Tajik, P., Barin, A., Movahedin, M., Zarnani, A., Hadavi, R., Moghaddam, G., Shoja, J., Jeddi-Tehrani, M., Ashrafi-Helan, J., Heidari-Vala, H., Torkabadi, E. and Qasemi-Panahi, B.(2010)Nestin, a neuroectodermal stem cell marker, is expressed by bovine sertoli cells. Comparative Clinical Pathology, 1-5.

van Pelt, A.M., Morena, A.R., van Dissel-Emiliani, F.M., Boitani, C., Gaemers, I.C., de Rooij, D.G. and Stefanini, M.(1996) Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. *Biology of Reproduction* **55**, 439-444.

Walker, W.H. and Cheng, J.(2005) FSH and testosterone signaling in Sertoli cells. *Reproduction* **130**, 15-28.

Wrobel, K. -H., Bickel, D., Kujat, R. and Schimmel, M.(1995) Evolution and ultrastructure of the bovine spermatogonia precursor cell line. *Cell and Tissue Research* **281**, 249-259.

Iranian Journal of Veterinary Science and Technology
Vol. 5, No. 1, 2013, 26-34

IJVST

اثر هورمون های FSH و تستوسترون بر میزان زنده مانی سلول های بنیادی اسپرماتوگونیال گاوی متعاقب روند انجماد و ذوب

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دریافت مقاله: ۱۳۹۱/۰۲/۰۲ پذیرش نهایی: ۱۳۹۱/۱۱/۱۸

چکیده

سلول های بنیادی عموما به عنوان سلول هایی تعریف می شوند که دارای قابلیت مشابه سازی و تمایز هستند. احتمالا بهترین روش برای نگهداری طولانی مدت سلول های بنیادی اسپرماتوگونی انجماد است. در این مطالعه، تاثیر هورمون های تحریک کننده ی فولیکول و تستسترون بر روی میزان زنده مانی سلول های بنیادی اسپرماتوگونی منجمد شده بعد از ذوب این سلول ها بررسی شده است. سلول های سرتولی و اسپرماتوگونی از گوساله های ۳-۵ ماهه جداسازی شده، و دو هورمون مذکور به هم کشتی سلول های بنیادی اسپرماتوگونی و سرتولی اضافه گردیده است. نتایج نشان داد که هورمون تحریک کننده فولیکول، میزان زنده مانی را در سلول های بنیادی اسپرماتوگونی منجمد شده نسبت به گروه‌های درمانی تستسترون و همچنین گروه کنترل افزایش داده است. در مجموع، استفاده از هورمون تحریک کننده فولیکول موجب فراهم شدن محیط کشتی مناسب برای سلول‌های بنیادی گوساله شده که می تواند میزان زنده مانی سلول های بنیادی اسپرماتوگونی منجمد شده را افزایش دهد.

واژگان کلیدی: انجماد، گوساله، FSH، تستوسترون